

**Figure 3. ATO inhibits anchorage-independent osteosarcoma growth.** Treatment of 143B and Saos2 cells with 3  $\mu$ M ATO reduced the number of colonies in soft agar at 14 days. An equivalent volume of vehicle was used as the control. These experiments were performed in triplicate with similar results (\* $P < 0.01$ ) (error bars represent mean [SD]).

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treatment (Figure 6C). These findings suggest that ATO promotes the accumulation of DNA damage by inhibiting Hedgehog signaling.

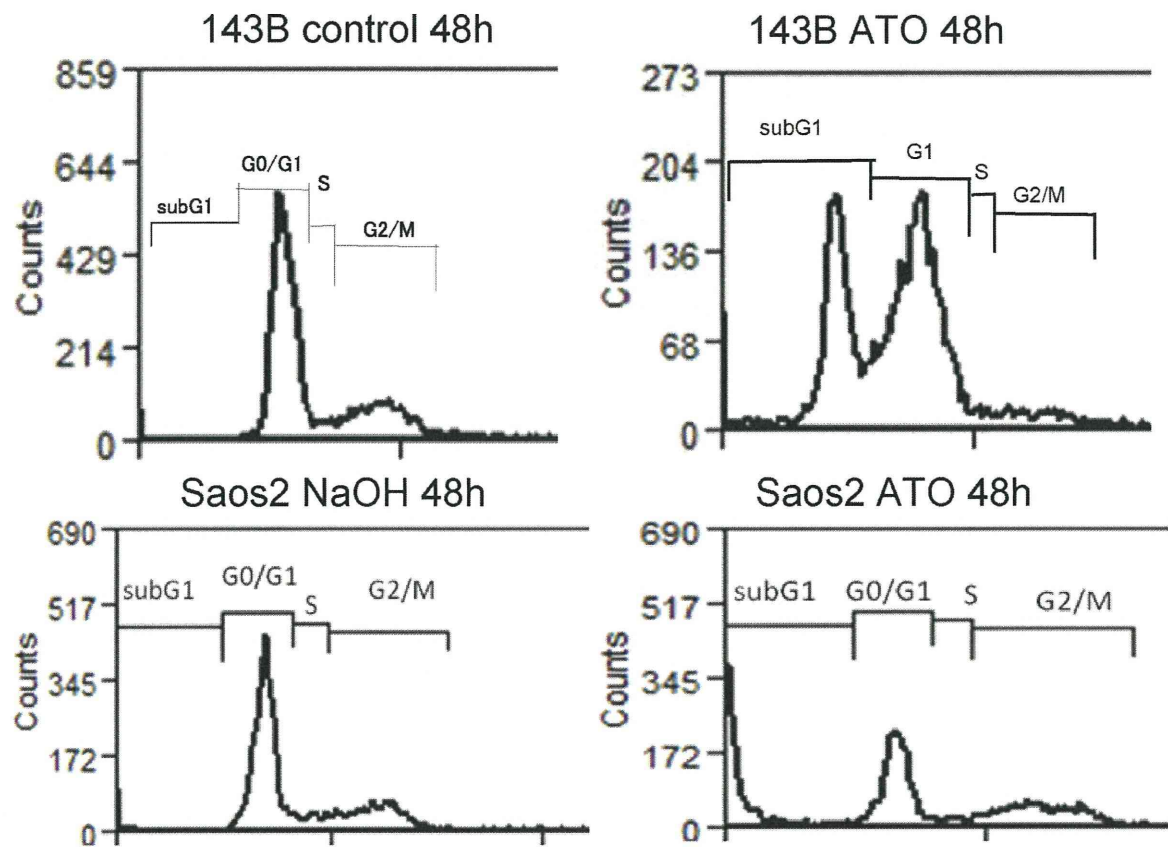
#### ATO prevents osteosarcoma growth in vivo

143B osteosarcoma cells were intradermally inoculated into nude mice, and palpable tumors were formed within 7 days. Then, ATO or an equivalent volume of vehicle was injected intraperitoneally. The injections were administered every day. Compared with vehicle treatment, treatment with ATO significantly prevented tumor growth (Figure 7). Kaplan-Meier analysis showed that ATO treatment provided a significant survival benefit (Figure 7A). TUNEL staining showed that ATO treatment induced apoptotic cell death. The number of apoptotic cells was significantly increased in ATO-treated tumors (Figure 7B).

#### Discussion

We and other researchers have previously reported that inhibition of the Hedgehog pathway prevented the growth of

osteosarcoma cells [9,10,29]. In particular, we showed that knockdown of GLI2 prevented osteosarcoma cell growth in vitro and in vivo [9]. ATO prevents Ewing sarcoma, medulloblastoma, and basal cell carcinoma growth by inhibition of GLI transcription [23–25]. To apply our previous findings in clinical settings, we examined the effects of ATO in human osteosarcoma. We showed that ATO prevents the transcription of GLI target genes and promotes apoptotic cell death in osteosarcoma cells as a result of accumulation of DNA damage. In addition, ATO re-induces the accumulation of DNA damage attenuated by recombinant Sonic Hedgehog treatment. These findings suggest that ATO inhibits the activation of Hedgehog signaling and promotes apoptotic cell death in osteosarcoma cells as a result of accumulation of DNA damage. In addition, our findings showed that ATO decreased the expression of Bcl-2 and Bcl-xL. GLI1 and GLI2 upregulate the transcription of Bcl-2 and Bcl-xL [30–33]. Inhibition of the Hedgehog pathway by ATO treatment may downregulate Bcl-2 and Bcl-xL to promote apoptotic cell death in osteosarcoma cells. Singh et al. reported that ABCG2, a drug transporter protein, is a direct transcriptional target of Hedgehog signaling [33]. These findings suggest that activation of Hedgehog



	SubG1	G1	S	G2/M
143B control	1.5±1.6 %	68.7±3.4 %	6.0±1.3 %	23.5±2.4 %
143B (ATO: 3 µM)	24.5±10.0 % **	45.2±21.7 %	2.9±1.1 % **	5.7±2.6 % *
Saos2 control	2.7±2.8 %	56.0±15.3 %	7.4±2.1 %	34.0±10.9 %
Saos2 (ATO: 3 µM)	25.7±6.4 % **	33.0±9.2 %	5.0±3.0 %	24.1±6.0 %

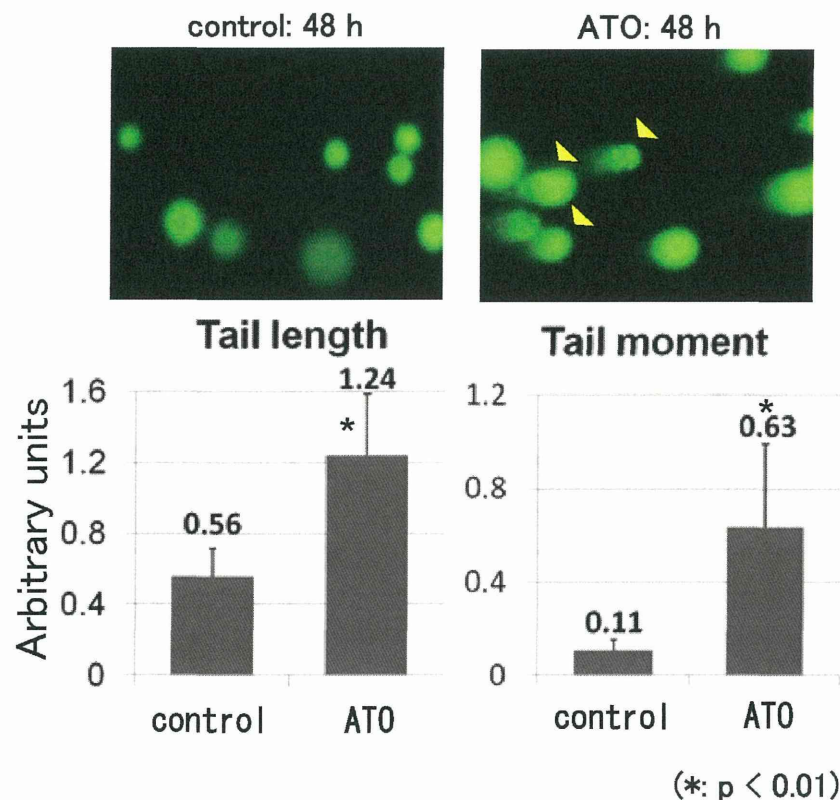
(\*: P &lt; 0.01, \*\*: P &lt; 0.05)

**Figure 4. ATO promotes apoptotic cell death in human osteosarcoma cells.** Human osteosarcoma cells were cultured with or without 1 µM ATO. An equivalent volume of vehicle was used as the control. Flow cytometric analysis was performed after ATO treatment for 48 h. ATO treatment significantly increased the Sub-G1 population of 143B and Saos2 cells. These experiments were performed in triplicate with similar results (\*P < 0.01, \*\*P < 0.05).

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signaling promoted the export of CDDP by the ABCG2 transporter and reduced the accumulation of DNA damage in osteosarcoma cells. Inhibition of the Hedgehog pathway by ATO treatment may be useful as an adjunct treatment to conventional chemotherapy for osteosarcoma. In addition,

several molecular mechanisms have been reported for inhibition of the Hedgehog pathway by ATO. Kim et al. reported that ATO prevented growth of medulloblastoma by reducing stability of GLI2 protein and ciliary accumulation of GLI2 [25]. Elspeth et al. reported that ATO prevents growth of cancer cell



**Figure 5. ATO elicits DNA damage in human osteosarcoma.** COMET assay was performed to detect DNA damage in single cells after ATO treatment. 143B cells were treated with ATO (3  $\mu$ M) or an equivalent volume of control vehicle for up to 48 h and analyzed by performing the COMET assay. Graphs represent DNA damage by tail length and tail moment, evaluated as described in the Materials and Methods section. These experiments were performed in triplicate with similar results (\* $P < 0.01$ ).

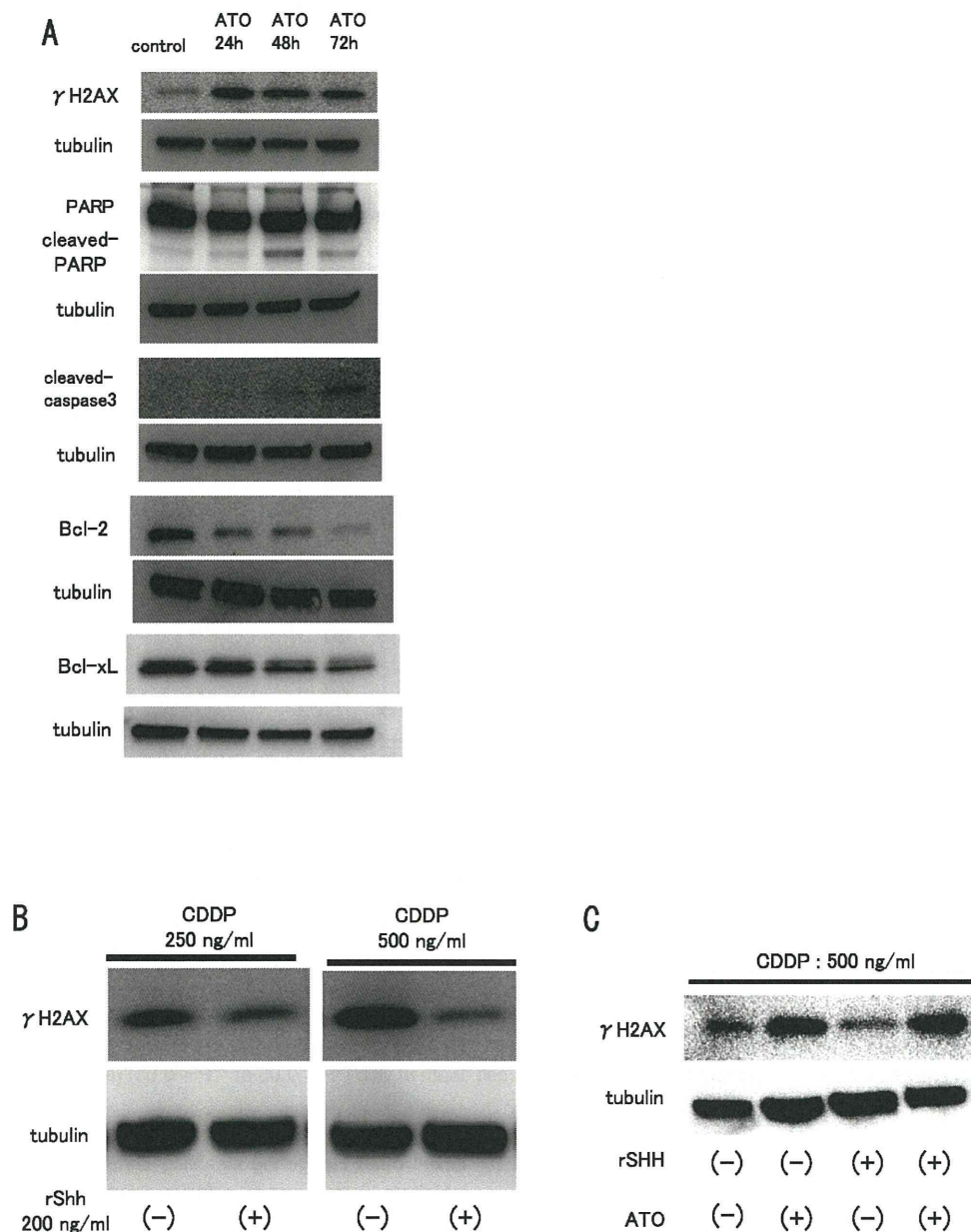
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lines and Ewing sarcoma by inhibiting GLI transcription through direct binding to GLI [23]. Although there were some discrepancies related to the mechanism of Hedgehog pathway inhibition by ATO, these studies independently suggest that ATO inhibits malignant tumor growth by inhibition of the Hedgehog pathway at the level of GLI transcription factors. These mechanisms may prevent osteosarcoma growth after ATO treatment. Because aberrant activation of the Hedgehog pathway has been implicated in several malignant tumors, the pharmaceutical industry has invested in the development of Hedgehog pathway inhibitors. SMO inhibitors have been evaluated in recent clinical trials [34,35]. However, treatment with SMO inhibitors showed a lack of efficacy in a portion of patients. Investigation of the underlying mechanism revealed that the patient tumors showed a mutation in SMO that prevented binding of the SMO inhibitors to SMO [15]. Several genes with potential mutations within SMO and downstream of SMO have been found [16–21,36]. In addition, non-Hedgehog pathway-mediated activation of GLI transcription has been

reported [37–41]. In this regard, direct GLI inhibition by ATO is likely to be useful for treating tumors with mutations within or downstream of SMO. For example, inhibition of GLI, but not SMO, inhibited tumor growth in myeloid leukemia, colon carcinoma, hepatocellular carcinoma, and osteosarcoma [9,42–44]. Originally, arsenic was used in the 17<sup>th</sup> century to treat leukemia. ATO has been approved for the treatment of intractable acute promyelocytic leukemia in Japan. Our findings suggest that ATO is one of the most suitable molecular target reagents for inhibiting the Hedgehog pathway in human osteosarcoma. We have now obtained approval from the ethics committee for clinical research, Kagoshima University, to use ATO for treating patients with intractable osteosarcoma.

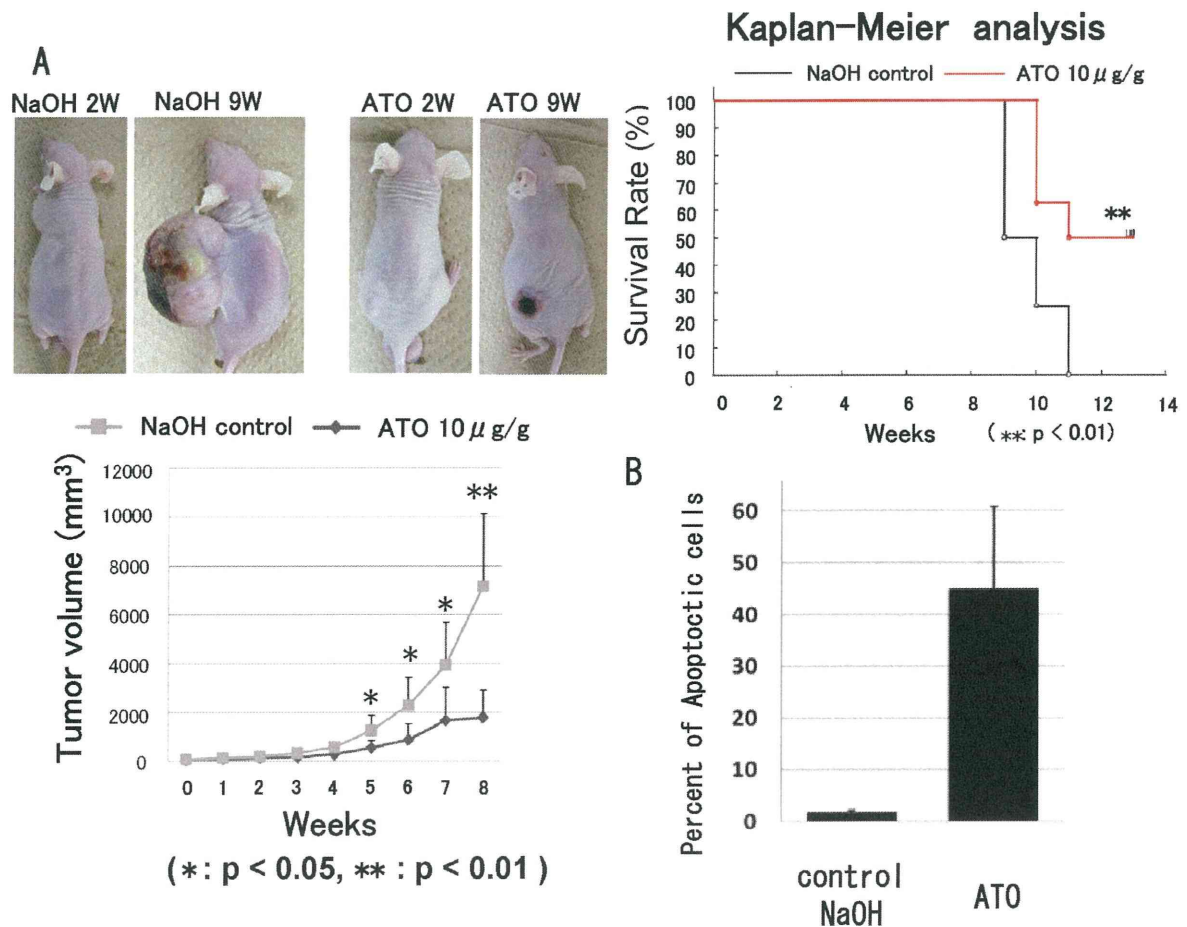
We examined whether the inhibitory effect of ATO on osteosarcoma growth is mediated, at least in part, by JNK or NF- $\kappa$ B [45–47]. As previously reported, treatment with ATO increased JNK phosphorylation. However, treatment with a JNK inhibitor did not prevent osteosarcoma growth. In contrast, treatment with ATO did not affect NF- $\kappa$ B activation. These





**Figure 6. ATO elicits DNA damage and apoptosis.** Human osteosarcoma cells were cultured with or without 3  $\mu$ M ATO. An equivalent volume of vehicle was used as the control. Western blot analysis was performed 48 h and 72 h after ATO treatment. (A) Western blot analysis revealed that ATO treatment increased the protein levels of  $\gamma$ H2AX, cleaved PARP, and cleaved caspase-3. ATO treatment decreased the protein levels of Bcl-2 and Bcl-xL. (B) Western blot analysis performed after cisplatin (CDDP) and recombinant human Sonic Hedgehog (rSHH) treatment showed that CDDP treatment upregulated the expression of  $\gamma$ H2AX. Addition of Sonic Hedgehog decreased the expression level of  $\gamma$ H2AX protein, which was upregulated by CDDP treatment. (C) Western blot analysis was performed following CDDP and recombinant human Sonic Hedgehog (rSHH) or ATO treatment. Addition of Sonic Hedgehog decreased the expression level of  $\gamma$ H2AX protein, which was upregulated by CDDP treatment. Addition of ATO restored the  $\gamma$ H2AX expression attenuated by rSHH treatment. These experiments were performed in triplicate with similar results.

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**Figure 7. ATO prevents osteosarcoma growth in vivo.** 143B cells ( $1 \times 10^6$ ) were subcutaneously inoculated into nude mice. Tumor volume was calculated weekly using the formula  $LW^2/2$  (where L and W represent the length and width of tumors). Seven days after inoculation, the tumor volume was set as 1 and was evaluated at different time points. (A) ATO treatment inhibited tumor growth as compared with control (\* $P < 0.05$  or \*\* $P < 0.01$ ) (error bars represent mean [SD]). Kaplan-Meier analysis revealed that ATO treatment provided a significant survival benefit (\*\* $P < 0.01$ ). (B) Apoptotic cell death in the tumors was analyzed by TUNEL staining, which showed that ATO treatment increased apoptotic cell death in vivo (\* $P < 0.05$  or \*\* $P < 0.05$ ) (error bar indicates SD).

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findings indicate that JNK or NF- $\kappa$ B activation does not affect the cytotoxicity of ATO in human osteosarcoma.

For in vivo examinations, we administered ATO intraperitoneally at 10 mg/kg body weight, as previously reported [25]. Kim et al. examined the ATO levels in mouse sera collected after ATO administration by injection at 10 mg/kg body weight. The peak concentration following intraperitoneal injection at 10 mg/kg was 2.6-fold higher than the peak plasma levels in human patients following intravenous ATO injection at a dose of 0.15 mg/kg body weight [48]. Area under the curve calculations revealed that the total exposure to ATO in mice at the 10 mg/kg dose was 2-fold higher than that in patients. To decrease the ATO concentration, combinations of drugs that

inhibit other Hedgehog signaling components, including SMO inhibitors, were used to achieve greater pathway inhibition at lower ATO concentrations [25]. In addition, Kim et al. reported that combined use of ATO and itraconazole, a commonly used antifungal that inhibits SMO by a mechanism distinct from that of cyclopamine and other known SMO antagonists, decreases the dose of ATO and itraconazole required to prevent medulloblastoma and basal cell carcinoma growth associated with acquired resistance to SMO antagonists [24].

In summary, our findings showed that ATO inhibits the Hedgehog pathway and human osteosarcoma cell growth in vitro and in vivo. The combined administration of conventional



anticancer agents or other Hedgehog pathway inhibitors with ATO may be valuable for treating osteosarcoma patients.

## Supporting Information

**Figure S1. Western blot analysis showed that ATO treatment decreased the expression of phosphorylated JNK.** Western blot analysis showed that ATO treatment did not affect the expression levels of NFκB and phosphorylated NFκB proteins. WST assay showed that JNK inhibitor did not affect the proliferation of osteosarcoma cells.

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(TIF)

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## Author Contributions

Conceived and designed the experiments: TS. Performed the experiments: S. Nakamura S. Nagano HN. Analyzed the data: YI MA TY SK TS. Contributed reagents/materials/analysis tools: MY. Wrote the manuscript: SK TS.

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**Cell Biology:**

**Human Immunodeficiency Virus Type 1  
Enhancer-binding Protein 3 Is Essential for  
the Expression of Asparagine-linked  
Glycosylation 2 in the Regulation of  
Osteoblast and Chondrocyte Differentiation**

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# Human Immunodeficiency Virus Type 1 Enhancer-binding Protein 3 Is Essential for the Expression of Asparagine-linked Glycosylation 2 in the Regulation of Osteoblast and Chondrocyte Differentiation<sup>\*,[5]</sup>

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**Background:** The mechanisms by which Hivep3 regulates the osteochondrogenesis remain elusive.

**Results:** Knockdown of Hivep3 down-regulated *Alg2* expression. *Alg2* suppressed osteoblast differentiation by inhibiting the activity of Runx2. *Alg2* silencing suppressed the expression of *Creb3l2* and chondrogenesis.

**Conclusion:** *Alg2* may be a modulator of osteochondrogenesis.

**Significance:** This is the first report to describe the association of an *Alg* gene with osteochondrogenesis.

Human immunodeficiency virus type 1 enhancer-binding protein 3 (*Hivep3*) suppresses osteoblast differentiation by inducing proteasomal degradation of the osteogenesis master regulator Runx2. In this study, we tested the possibility of cooperation of Hivep1, Hivep2, and Hivep3 in osteoblast and/or chondrocyte differentiation. Microarray analyses with ST-2 bone stroma cells demonstrated that expression of any known osteochondrogenesis-related genes was not commonly affected by the three Hivep siRNAs. Only *Hivep3* siRNA promoted osteoblast differentiation in ST-2 cells, whereas all three siRNAs cooperatively suppressed differentiation in ATDC5 chondrocytes. We further used microarray analysis to identify genes commonly down-regulated in both MC3T3-E1 osteoblasts and ST-2 cells upon knockdown of *Hivep3* and identified asparagine-linked glycosylation 2 (*Alg2*), which encodes a mannosyltransferase residing on the endoplasmic reticulum. The *Hivep3* siRNA-mediated promotion of osteoblast differentiation was negated by forced *Alg2* expression. *Alg2* suppressed osteoblast differentiation and bone formation in cultured calvarial bone. *Alg2* was immunoprecipitated with Runx2, whereas the combined transfection of Runx2 and *Alg2* interfered with Runx2 nuclear localization, which resulted in suppression of Runx2 activity. Chondrocyte differentiation was promoted by *Hivep3* overexpression, in concert with increased expression of *Creb3l2*, whose gene product is the endoplasmic reticulum stress inducer crucial for chondrogenesis. *Alg2* silencing suppressed *Creb3l2* expression and chondrogenesis of ATDC5 cells, whereas infection of *Alg2*-expressing virus promoted chondrocyte maturation in cul-

tured cartilage rudiments. Thus, *Alg2*, as a downstream mediator of Hivep3, suppresses osteogenesis, whereas it promotes chondrogenesis. To our knowledge, this study is the first to link a mannosyltransferase gene to osteochondrogenesis.

In skeletal development and bone remodeling, osteoblasts play major roles not only in bone formation but also in inducing the differentiation of bone-resorbing osteoclasts (1, 2). Runx2 is a critical transcription factor in osteoblast differentiation, as evidenced by Runx2 knock-out mice, which exhibit a complete lack of both intramembranous and endochondral ossification due to the absence of osteoblasts (3). Cleidocranial dysplasia, a human autosomal dominant inherited disorder of bone development, is characterized by hypoplasia of clavicles and abnormalities in cranial and facial bones and is caused by mutations in the *Runx2* gene (4, 5). Some genes, e.g. LDL receptor-related protein 5 (*Lrp5*), sclerostin (*Sost*), and human immunodeficiency virus type 1 enhancer-binding protein 3 (*Hivep3*), have been found to control osteoblast function in the adult human and/or mouse during postnatal skeletal remodeling (6–10).

Hivep3, also known as Schnurri-3, Zas3, and Krc, is a member of three mammalian homologs of the Hivep/Schnurri family of large zinc finger proteins. Hivep proteins have been studied for their roles in the regulation of an assortment of genes, including those encoding collagen type IIA,  $\alpha$ A-crystallin,  $\beta$ -interferon, and HIV genes (11). Hivep2 can indirectly interact with the peroxisome proliferator-activated receptor  $\gamma$ 2 (*Pparg2*) promoter to promote adipogenesis, through binding to Smad1, an intracellular mediator of bone morphogenetic protein (BMP)<sup>2</sup> signaling. Hivep2 can also dock to CCAAT/enhancer-binding protein  $\alpha$  (*C/ebp $\alpha$* ) to interact

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[5] This article contains supplemental Tables 1–5.

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<sup>2</sup> The abbreviations used are: BMP, bone morphogenetic protein; ER, endoplasmic reticulum; ALP, alkaline phosphatase; ECM, extracellular matrix; CDG, congenital disorders of glycosylation; qRT, quantitative RT; ALG, asparagine-linked glycosylation.



## Hivep3-dependent Alg2 Expression Inhibits Osteogenesis

with a CCAAT site on the distal part of the *Pparg* gene (12). Mice lacking Hivep3 demonstrate adult-onset osteosclerosis with increased bone volume due to enhanced osteoblast activity (10). Hivep3 promotes proteasomal degradation of the Runx2 protein through recruitment of the E3 ubiquitin ligase Wwp1 to Runx2 (10). A D-domain motif within Hivep3 mediates the interaction with and inhibition of ERK mitogen-activated protein kinase (MAPK), thereby inhibiting Wnt/Lrp5 signaling through regulation of the activity of a downstream mediator glycogen synthase kinase 3- $\beta$  (Gsk3 $\beta$ ). This interaction results in the suppression of subsequent osteoblast differentiation (13). In addition, Hivep3 indirectly promotes osteoclastogenesis by promoting osteoblastic expression of receptor activator of nuclear factor- $\kappa$ B ligand (Rankl), a crucial factor for osteoclast differentiation (14). Hivep3 also cell-autonomously promotes osteoclastogenesis by inducing the expression of *Nfatc1*, a master transcription factor in osteoclast differentiation, by interacting with Traf6 to enhance its activity while forming a complex with c-Jun to activate the *Nfatc1* promoter (15). Thus, Hivep3 controls both bone formation and resorption at multiple steps to maintain normal bone mass. However, whether Hivep3 controls gene expression in osteoblasts to regulate osteoblast activity is unclear.

In contrast to *Hivep3* knock-out mice, mice lacking *Hivep2* exhibited decreased cortical bone volume and increased cancellous bone mass (16), suggesting different roles for Hivep2 and Hivep3 in the skeleton. Combined ablation of *Hivep2* and *Hivep3* in mice resulted in synergistically increased trabecular bone volume, demonstrating a redundancy between the two proteins in the regulation of postnatal bone mass (17). Interestingly, in the double knock-out mice, the growth plate cartilage of the long bones was uncoupled with bone phenotype, with significantly delayed maturation of chondrocytes resulting in chondrodysplasia (17), suggesting a role for Hivep proteins in the promotion of chondrocyte differentiation. However, the mechanism by which Hivep proteins affect chondrogenesis remains unknown. In addition, to date, no information has been reported on the possible role of Hivep1 in osteogenesis and/or chondrogenesis.

In this study, *in vitro* analysis showed that, among the three Hivep proteins, only Hivep3 was inhibitory and that the others promoted osteoblast differentiation. In contrast, all three Hivep genes seemed to support chondrocyte differentiation in BMP-2-induced ATDC5 cells, suggesting their redundancy in chondrogenesis. We found that asparagine-linked glycosylation 2 (*Alg2*) is commonly down-regulated in BMP-2-induced osteoblast differentiation in both MC3T3-E1 and ST-2 cells. *Alg2* inhibited Runx2 activity without altering its protein level, resulting in suppression of osteoblast differentiation. Interestingly, in chondrogenesis of ATDC5 cells, Hivep3 induced the expression of cAMP-responsive element binding-protein 3-like 2 (*Creb3l2*), an endoplasmic reticulum (ER) stress transducer crucial for chondrogenesis (18), suggesting a possible role for Hivep3 in physiological mild ER stress. *Alg2* was also decreased by *Hivep3* knockdown in ATDC5 chondrocytes, whereas silencing of *Alg2* suppressed the expression of *Creb3l2* and chondrogenesis. To our knowledge, this study is the first to show a linkage between an asparagine-linked glycosylation mannosyltransferase gene and osteochondrogenesis.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Differentiation**—The mouse calvarial bone-derived osteoblast cell line, MC3T3-E1 (clone 4), and the mouse chondrogenic fibroblast cell line, C3H10T1/2, were obtained from the ATCC. The mouse bone marrow stromal cell line ST-2 and the mouse chondrogenic cell line ATDC5 were obtained from the RIKEN BioResource Center. MC3T3-E1 cells were cultured in minimum essential medium  $\alpha$  (Invitrogen) containing 10% fetal bovine serum (FBS). ST-2 cells were cultured in RPMI 1640 medium (Sigma) containing 10% FBS. ATDC5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) (Invitrogen) containing 5% FBS. C3H10T1/2 cells were cultured in basal medium Eagle's (Sigma) with 2 mM L-glutamine and 10% FBS. COS-7 cells were purchased from RIKEN BioResource Center and maintained in DMEM supplemented with 10% FBS. All cell culture medium contained 100 units/ml penicillin G and 100  $\mu$ g/ml streptomycin. Cell differentiation was induced by the addition of recombinant human BMP-2 (PeproTech) at a concentration of 300 ng/ml. Micromass culture of ATDC5 cells was performed as described previously (19) to accelerate the maturation of chondrocyte differentiation.

**Alkaline Phosphatase (ALP) and Alcian Blue Staining**—The activity of ALP secreted into the extracellular matrix (ECM) of cultured cells was visualized with an ALP staining kit (85L-3R, Sigma). Cartilaginous glycosaminoglycans produced in the ECM by cultured cells were stained with Alcian blue 8GX (Sigma).

**RNA Interference**—Dharmacon siRNA ON-TARGETplus SMARTpool, a mixture of four independent siRNAs against mouse *Hivep1*, *Hivep2*, *Hivep3*, and *Alg2*, and the control reagent were purchased from Thermo Scientific. siRNAs were transfected into cells using Lipofectamine RNAiMax (Invitrogen).

**Real Time Quantitative PCR**—Cells were lysed with TRIzol reagent (Invitrogen) to purify RNA, and 1  $\mu$ g of total RNA was subjected to reverse transcription with the Verso cDNA kit (Thermo Scientific). The relative amounts of the gene transcripts were determined by real time quantitative PCR using SYBR premix Ex TaqII (Takara) and the Thermal Cycler Dice TP850 system (Takara). PCRs were performed in duplicate per sample, and the measured expression level of each gene was normalized to that of *Hprt1*. The sequence information for the primers used is listed in supplemental Table 1. All primer sets are for mouse genes, except for the m/h*Hivep3* primer set, which can be used to amplify both the mouse and human *Hivep3* genes. For evaluation of the tissue distribution of the Hivep genes and *Alg2* *in vivo*, tissues were harvested from 3-month-old mice, and mRNA was purified with TRIzol reagent before subjecting samples to qRT-PCR.

**Microarray Analysis**—Cells transfected with siRNA overnight were further incubated with BMP-2 for 2 days before being lysed with TRIzol reagent for mRNA purification. mRNA samples were cleaned up using the RNeasy MinElute Cleanup kit (Qiagen) and analyzed on a Mouse Gene 2.0 ST Array (Affymetrix) by BioMatrix Research.